F ENT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing: 21 October 1999 (21.10.99)	
International application No.: PCT/EP98/07553	Applicant's or agent's file reference: DIR 0549
International filing date: 24 November 1998 (24.11.98)	Priority date: 25 November 1997 (25.11.97)
Applicant: AGSTERIBBE, Etienne et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

07 May 1999 (07.05.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

F. TENT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

BREEPOEL, Peter, Maria
Octrooibureau Zoan B.V.
C.J. van Houtenlaan 36
NL-1381 CP Weesp
PAYS-BAS

Date of mailing (day/month/year)
30 May 2000 (30.05.00)

Applicant's or agent's file reference
DIR 0549

IMPORTANT NOTIFICATION

International application No.
PCT/EP98/07553

International filing date (day/month/year)
24 November 1998 (24.11.98)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address
AGSTERIBBE, Etienne (deceased)

State of Nationality: NL
State of Residence: NL
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
AGSTERIBBE-SERVAAS, Irène
Adelheidstraat 16
NL-9717 AS Groningen
Netherlands

State of Nationality: NL
State of Residence: NL
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:
AGSTERIBBE-SERVAAS, Irène shall be added to the records as the heiress of the deceased applicant/inventor. AGSTERIBBE-SERVAAS, Irène shall be applicant for US only.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
A. Karkachi
Telephone No.: (41-22) 338.83.38

F. ENT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

BREEPOEL, Peter, Maria
Octrooibureau Zoan B.V.
C.J. van Houtenlaan 36
NL-1381 CP Weesp
PAYS-BAS

Date of mailing (day/month/year)
30 May 2000 (30.05.00)

Applicant's or agent's file reference
DIR 0549

IMPORTANT NOTIFICATION

International application No.
PCT/EP98/07553

International filing date (day/month/year)
24 November 1998 (24.11.98)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address
AGSTERIBBE, Etienne

State of Nationality: NL
State of Residence: NL
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
AGSTERIBBE-SERVAAS, Irène
Adelheidstraat 16
NL-9717 AS Groningen
Netherlands

State of Nationality: NL
State of Residence: NL
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:
Applicant/inventor in Box 1 is deceased. AGSTERIBBE-SERVAAS is the heiress of the deceased inventor and applicant for the United States of America.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
A. Karkachi
Telephone No.: (41-22) 338.83.38

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 39/39 // 39:145

A1

(11) International Publication Number: WO 99/26654

(43) International Publication Date: 3 June 1999 (03.06.99)

(21) International Application Number: PCT/EP98/07553

(22) International Filing Date: 24 November 1998 (24.11.98)

(30) Priority Data:
97203671.9 25 November 1997 (25.11.97) EP

(71) Applicants *(for all designated States except US)*: DUPHAR INTERNATIONAL RESEARCH B.V. [NL/NL]; Patent Dept., C.J. van Houtenlaan 36, NL–1381 CP Weesp (NL). UNIVERSITEIT VAN GRONINGEN [NL/NL]; Oude Boteringestraat 14, NL–7912 GL Groningen (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: AGSTERIBBE, Etienne [NL/NL]; Duphar International Research B.V., C.J. van Houtenlaan 36, NL–1381 CP Weesp (NL). BRANDS, Rudi [NL/NL]; Duphar International Research B.V., C.J. van Houtenlaan 36, NL–1381 CP Weesp (NL). DE HAAN, Lolke [NL/NL]; Duphar International Research B.V., C.J. van Houtenlaan 36, NL–1381 CP Weesp (NL). VAN SCHARRENBURG, Gustaaf, Johan, Marie [NL/NL]; Duphar International Research B.V., C.J. van Houtenlaan 36, NL–1381 CP Weesp (NL). VERWEIJ, Willem, Ronald [NL/NL]; Duphar International Research B.V., C.J. van Houtenlaan 36, NL–1381 CP Weesp (NL). WILSCHUT, Jan, Christiaan [NL/NL]; Duphar International Research B.V., C.J. van Houtenlaan 36, NL–1381 CP Weesp (NL).

(74) Agents: BREEPOEL, Peter, Maria et al.; Octrooibureau Zoan B.V., C.J. van Houtenlaan 36, NL–1381 CP Weesp (NL).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: VACCINES WITH AN LTB ADJUVANT

(57) Abstract

The present invention relates to a vaccine containing at least one particulate immunogen and an adjuvanting amount of B subunits of heat–labile enterotoxin characteristic of *E. Coli*. More in particular, this invention relates to vaccines wherein the adjuvanting LTB is free from contaminating A subunits or holotoxin. To this end, preferably, use is made of LTB prepared by recombinant DNA techniques. The particulate immunogens can relate to or can be derived from e.g. viruses, bacteria or fungi. This vaccine is particularly suitable for the induction of a protective response against said particulate immunogen upon mucosal (e.g. intra–nasal) administration. It was found that such administration results in both systemic and mucosal protection against the pathogen to which the particulate immunogen relates.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

VACCINES WITH AN LTB ADJUVANT

The present invention relates to a vaccine containing the B subunits of heat-labile enterotoxin (LTB) of *Escherichia coli* (*E. coli*) as a mucosal immunoadjuvant. The invention relates in particular to a vaccine of this type to prevent influenza infections in humans. However, the invention is not restricted to application in influenza vaccines.

It is the object of vaccination against infectious diseases to prevent or at least restrain infection of the vaccinated subject by stimulating an immune response against the infectious agent through introduction of an antigen formulation derived from the particular pathogen. Ideally, the induced immune response should consist of two components, a humoral response (the production of antigen-specific antibodies) and a cellular response (the generation of specific cytotoxic T lymphocytes, capable of eliminating cells infected by the pathogen).

Many vaccination procedures involve the administration of a formulation containing inactivated or attenuated whole pathogen. However, for certain pathogens there is a considerable disadvantage to vaccination with whole pathogen, since such preparations, even though they are usually highly immunogenic, may have undesirable side effects. This explains the current trend towards the use of well-defined subunit vaccines or synthetic vaccines, substantially lacking the adverse side effects of the whole infectious agent. However, compared to whole pathogen, subunit vaccines or synthetic vaccines are often not very immunogenic, at least in the absence of an added adjuvant.

Adjuvants are substances or materials administered in conjunction with the antigen so as to stimulate the immune response against that antigen. There is a need for appropriate adjuvants which would boost the immune response against subunit antigens or synthetic antigens without causing undesirable side effects.

Influenza vaccine formulations have contained for a long time, and in some cases still contain, inactivated or attenuated whole virus. Such formulation may have considerable side effects, most notably fever and reactions at the site of injection. Nowadays,

vaccination is usually done with a subunit formulation. This subunit vaccine, which causes less side reactions, only contains the two major surface antigens of the virus, the hemagglutinin (HA) and the neuraminidase (NA), in a more or less purified form. In most current vaccine formulations there is no added adjuvant present.

The inactivated or attenuated whole influenza virus vaccine as well as the subunit vaccine are usually administered via a single intramuscular (i.m.) injection. The protection against influenza infection, achieved by either vaccination procedure, is comparatively low, particularly in elderly people. The relatively low efficacy of vaccination against influenza is due in part to the high antigenic variability of the virus. However, there is reason to believe that the protection against influenza infection by vaccination can be improved by stimulation and/or modification of the immune response against the antigen.

In the case of influenza, or in general in cases in which the infection is contracted via the respiratory tract, strategies for improved vaccination efficacy should aim at the generation of not only an adequate T-cell-dependent IgG response in the circulation, but also at a local immune response (secretory IgA) in the lungs and nasal cavity as a first line of defence against invading infectious virus. Furthermore, a cellular immune response (cytotoxic T-cells) might also be important, particularly in restricting the infection. It has been demonstrated that administration of influenza vaccine via i.m. injection (the current route of administration) does not result in a local IgA response in the respiratory tract.

The present invention relates to the surprising finding that the presence of LTB in an intranasal vaccine formulation not only stimulates the IgG response in the circulation, relative to i.m. immunisation with the adjuvant-free immunogen vaccine, but also generates a local IgA response in the respiratory tract.

The intact heat-labile enterotoxin (LT), and its close relative cholera toxin (CT), are composed of one A subunit and a pentameric ring structure consisting of five identical B subunits. The A subunit has enzymatic, ADP-ribosylation, activity and attributes the toxic activity to the toxins. In the intestinal epithelium the A subunit

induces persistent synthesis of second messenger cAMP, resulting in excessive electrolyte and concomitant fluid secretion to the lumen of the gut.

LT and CT are powerful mucosal immunogens. Upon local mucosal administration these molecules give rise to not only induction of a systemic antibody response directed against the toxin, but also to production of locally secreted antibodies, notably secretory IgA (S-IgA). LT and CT are also powerful mucosal immunoadjuvants. That is, when co-administered with an unrelated other immunogen, LT or CT may stimulate the systemic and mucosal antibody response against that immunogen. However, the toxicity of LT and CT has thusfar essentially precluded the use of LT or CT in human vaccine formulations.

In attempts to separate the toxic from the immune-stimulatory activities of LT or CT, detoxified mutants of the toxins, or the unmodified isolated pentameric B subunit (LTB or CTB, respectively), have been examined for their immunoadjuvant activity. Clearly, because the toxic ADP-ribosylation activity of the toxins resides in the A subunit, the presence of even trace amounts of unmodified A subunit or of LT or CT holotoxin in a human vaccine is highly undesirable.

The use of LTB as an adjuvant for influenza antigens has been investigated by Tamura and co-workers (Hirabashi et al.: Vaccine **8**: 243-248 [1990]; Kikuta et al.,: Vaccine **8**: 595-599 [1990]; Tamura et al. J.: Immunology **3**: 981-988 [1992]; Tamura et al.: Vaccine **12**: 419-426 [1994]; Tamura et al.: Vaccine **12**: 1083-1089 [1994]). In these studies, based on the use of soluble influenza virus hemagglutinin (HA) vaccine, extracted and purified from influenza virus by treatment with Tween/ether according to Davenport et al (J. Lab. & Clin. Med. **63**(1): 5-13 [1964], it was established that LTB, free of A subunit, lacks mucosal immunoadjuvant activity when administered intra-nasally in conjunction with the soluble HA antigen to mice. It was further demonstrated that the presence of trace amounts of holotoxin, for example residual holotoxin remaining in B subunit preparations isolated from holotoxin, restores the expression of adjuvant activity of LTB towards the soluble HA antigen. More in particular, when LTB from recombinant sources (and therefore, completely free of even the smallest trace amounts of A subunit) was used, a trace of holotoxin

had to be added in order for the LTB to exert mucosal activity upon intranasal co-administration with the soluble HA antigen.

Surprisingly, it was found that isolated LTB from recombinant origin and therefore completely free of A subunit, does possess powerful immunoadjuvant activity depending on the nature or presentation form of the intranasally co-administered immunogen.
For example, adjuvant activity towards freely mixed small soluble antigens, such as ovalbumin or the soluble ectodomain of the envelope glycoprotein of human immunodeficiency virus (gp120), is low and often undetectable. On the other hand, it was found that LTB does exert very powerful adjuvant activity towards freely mixed large aggregated or particulate immunogens. These immunogens include influenza virus subunit antigen and keyhole limpet hemocyanin (KLH).

Accordingly, the present invention is concerned with a vaccine containing at least one particulate immunogen and an adjuvanting amount of LTB completely free of A subunit or toxic LT holotoxin.

As defined herein, "particulate" means any association of viral, bacterial, or fungal antigens characteristic of the respective micro-organisms. More in particular, the term "particulate immunogen" comprises aggregates, clusters, micelles, virosomes, rosettes, virus-like immunogen particles, and the like.

In the vaccine according to the present invention, in particular, LTB prepared from recombinant DNA technology can be utilised. The immunogen or immunogens may be derived from infective agents, such as viruses or bacteria.

Vaccines which apply to the above description were found not only to induce systemic immunoglobulin (e.g. IgG) against the immunogen upon mucosal (e.g. intra nasal) administration, but were also found to induce local secretion of IgA.

This latter property is particularly favourable for immunisation against diseases which are transmitted by mucosal infection with viruses (such as influenza virus, herpes virus, papilloma virus) or bacteria (like *Chlamydia*, pneumococs), or fungi.

A particular advantage of mucosal administration is the ease of vaccine application, which, furthermore, circumvents potential needlefobia with vaccinees receiving an intramuscular immunisation.

Although, for example in the case of influenza infection, high serum IgG titres are important for preventing systemic spread of the virus and protection of the lungs against infection, local S-IgA antibodies are crucial as a first line of defence for protection of the upper respiratory tract.

It has been reported that mucosal vaccination by i.n. administration of inactivated influenza virus in the absence of a mucosal adjuvant was not successful (Clancy: Drugs **50**: 587-594 [1995]; Katz et al.; J. Infect. Dis. **175**: 352-369 [1997]), probably because direct administration of an antigen to mucosal tissue will not result in an S-IgA response. Co-administration of a mucosal adjuvant seems to be a prerequisite to induce a local immune response against an immunogen. Remarkably, it was found that by i.n. immunisation according to the present invention the so-called common mucosal immune system is activated which results in secretion of S-IgA not only at the site op application (i.n.) but also in distant mucosal tissues (e.g. in the vaginal mucosal tissue).

Vaccines according to the present invention may contain immunogens of e.g. viral or bacterial origin, such as bacterial antigens, viral subunits (optionally inactivated) split viruses (optionally inactivated) inactivated viruses or bacteria, or attenuated (e.g. cold-adapted) live viruses, in a particulate form.

The LTB used according to the present invention is strictly free of toxic LTA or toxic holotoxin. Preferably, the LTB is prepared by recombinant DNA technology. Free of toxic LTA in the present context means strictly LTA-free.

In the vaccine according to the present invention the LTB can be used freely admixed with the particulate antigen - a covalent coupling between the antigen and the adjuvant can be established, however, is not needed to attain adequate adjuvant effect.

Apart from LTB and one or more immunogens the vaccine according to the present invention may contain an aqueous solvent, in particular a buffer, more in particular PBS (phosphate-buffered saline) as well as a stabiliser (e.g. PEG or methyl cellulose), and or glucose.

The components of the vaccine according to the present invention may be freeze dried or in a liquid form.

The vaccine according to the present invention may be present e.g. in bulk, or in an ampoule, or in a syringe, or in a nebuliser.

The vaccine according to the present invention may be administered by subcutaneous, or intramuscular, or intra-bronchial, or intra-nasal or intra-vaginal application or per os.

EXAMPLE 1

PREPARATION OF RECOMBINANT LTB AND INFLUENZA SUBUNIT ANTIGEN

RECOMBINANT LTB

Recombinant LTB genes and recombinant LTB molecules, as mentioned in the present invention, may be derived from genes encoding LT-I molecules from e.g. a porcine or a human source. The porcine LT (pLT) gene was subcloned in the pUC18 vector (Vieira and Messing: Gene **19**: 259-268 [1982]) using PCR techniques (DeHaan et al.: Vaccine **14**: 260-266 [1996]). The EWD299 vector, originally described by Dallas et al. (J. Bacteriol. **139**: 850-858 [1979]) was used as a template in the PCR reaction. The primary pLT sequence of this construct was found to be exactly in accordance with the primary pLT sequence as submitted in the EMBL sequence databank, as verified by DNA sequencing. From the pUC18-pLT construct,

the pLTB gene was subcloned in the pROFIT expression vector, which contains a temperature inducible λPR promoter(van der Linden et al.: Eur. J. Biochem. **204**: 197-202 [1992]).

E. coli MC1061 was used as a host strain for the pROFIT plasmid constructs. Bacteria were grown on Luria-Bertani medium containing 50 μg of kanamycin per ml. Induction of pLTB expression was obtained by raising the culture temperature of log-phase MC1061 cultures harboring the pROFIT-LTB vector from 28 to 42 degrees Celsius as described by De Haan et al. (*supra*).

pTLTB, a pKK-derived expression vector (Pharmacia Ltd.) encoding human LTB (i.e. an LTB gene derived from an LT gene isolated from an E. coli bacterium enterotoxigenic in humans) was obtained from Tamura and co-workers. DNA sequencing revealed 3 amino acid substitutions in the mature human LTB (hLTB) compared to pLTB (Thr4 to Ser, Glu46 to Ala, and Lys102 to Glu). *E. coli* strain JM101 was used as a host for pTLTB. Bacteria were grown on LB medium containing 100 μg of ampicillin per ml. Induction of hLTB expression was obtained by addition of IPTG to log-phase cultures of JM101 harboring pTLTB to a final concentration of 5 mM.

For purification of pLTB and hLTB, overexpressing bacteria were harvested, and then lysed by sonication. Subsequently, cell debris was removed by ultracentrifugation. Crude cell extracts containg recombinant pLTB or hLTB were then applied to an immobilised D-galactose (Pierce) column. After extensive washing, recombinant purified pLTB or hLTB were obtained by elution with D-galactose as previously described by Uesaka et al. (Microb. Path. **16**: 71-76 [1994]). Both recombinant pLTB and hLTB were found to retain optimal GM1-binding properties in a GM1 capture ELISA, as described previously (DeHaan et al.: Vaccine **14**: 260-266 [1996]). Column fractions containing purified protein were pooled, dialysed against PBS and stored at 4°C.

INFLUENZA SUBUNIT ANTIGEN

The influenza subunit antigen was prepared from B/Harbin/7/94 virus (B/Harbin) or A/Johannesburg/33/94 (A/Johannesburg) grown on embryonated chicken eggs according to the method described by Bachmayer et al. (Patent specification

GB 1 498 261 of January 18, 1978) and by Chaloupka et al. (Eur. J. Microbiol. Infect. Dis. **15**: 121-127 [1996]). This method comprises the steps of treatment of the formaldehyde-inactivated viruses with a suitable cationic detergent, separation of the released antigens (hemagglutinin and neuraminidase) from the virus residual core. This method leads to particulate, i.e. micelle-like exposition of the antigens after removal of the detergent.

The potency of the subunit antigen preparations, expressed as μg per ml, was determined in a single-radial diffusion test according to Wood et al. (J. Biol. Stand. **5**: 237-241 [1977]).

EXAMPLE 2

SYSTEMIC ANTIBODY RESPONSE TO INFLUENZA SUBUNIT VACCINE

Groups of four mice were immunised i.n. without anaesthesia with 5 μg of influenza subunit antigen derived from either B/Harbin or A/Johannesburg virus prepared according to the method described in EXAMPLE 1. The antigen was given either alone (HA) or together with 2.0 μg of pLTB (pLTB), in all cases in a volume of 20 μl on days 0, 7 and 14. Control mice received the same volume of PBS. Mice were sacrificed on day 28. Serum IgG antibody response was determined in a direct ELISA.

Figure 1 shows the observed serum IgG antibody responses against HA B/Harbin (solid bars) and HA A/Johannesburg (open bars).

Nasal administration of the subunit antigen without adjuvant gave a poor systemic antibody response, whereas supplementation of the subunit antigen with pLTB enhanced the serum antibody response by more than two orders of magnitude. Differences between the responses of mice immunised with B/Harbin and A/Johannesburg were not significant.

These results show that non-toxic pLTB is a powerful adjuvant capable of inducing high systemic antibody responses towards i.n. administered influenza subunit antigen.

EXAMPLE 3

COMPARISON OF SYSTEMIC ANTIBODY RESPONSES WITH HUMAN AND PORCINE LTB

Groups of 4 mice were immunised i.n. without anaesthesia with 5 μg of influenza subunit antigen derived from B/Harbin influenza virus prepared according to the method described in EXAMPLE 1.

The antigen was given either alone (NONE) or together with either 2.0 μg of pLTB (pLTB) or 2.0 μg of hLTB (hLTB), in all cases in a volume of 20 μl on days 0, 7 and 14. Control animals received PBS. Mice were sacrificed on day 21. Serum IgG antibody response was determined in a direct ELISA on day 21.

Figure 2 shows the observed serum IgG antibody response against HA B/Harbin. Nasal administration of subunit antigen without adjuvant again gave a poor systemic antibody responses, whereas supplementation of subunit antigen with pLTB and with hLTB enhanced the serum antibody response to the same extent by more than two orders of magnitude. The observed differences between pLTB and hLTB treated animals were non-significant.

EXAMPLE 4

INDUCTION OF LOCAL MUCOSAL ANTIBODY RESPONSE TO INFLUENZA SUBUNIT VACCINE

In order to investigate the ability of pLTB to evoke influenza HA-specific S-IgA responses, nasal washes of the mice from EXAMPLE 2 were analysed for the presence of influenza -specific IgA antibodies. Nasal washes were obtained by flushing 0.5 ml of PBS retrograde via the nasopharynx to the upper part of the trachea, flushing back, and collecting the lavage fluid at the nostrils.

The results are shown in Figure 3.

The data show that recombinant pLTB induced strong local S-IgA responses against HA. The two different influenza subunit antigens gave similar results.

EXAMPLE 5

COMPARISON OF MUCOSAL ANTIBODY RESPONSES WITH HUMAN AND PORCINE LTB

In order to compare the capacities of pLTB and hLTB to enhance nasal HA-specific antibody responses, nasal washes of the mice from EXAMPLE 3 were taken as described above and analyzed for the presence of HA-specific S-IgA on day 21. Figure 4 shows that both pLTB and hLTB induced brisk nasal HA-specific antibody responses. Moreover, the responses obtained with pLTB and hLTB were comparable in magnitude, demonstrating that both molecules have comparable adjuvant properties.

EXAMPLE 6

INDUCTION OF GENITAL MUCOSAL ANTIBODY RESPONSE TO INFLUENZA SUBUNIT VACCINE APPLIED I.N.

In order to investigate the ability of recombinant pLTB to evoke influenza HA-specific S-IgA responses at mucosal sites other than the site of administration, the induction of influenza-specific S-IgA antibodies in the genital tract after i.n. immunisation in the mice from EXAMPLE 2 was investigated. Lavages of the urogenital tract were conducted by introducing and withdrawing a 100 µl volume of PBS ten times into the vagina using a pipette tip. Mucosal washes were stored at 4 °C until determination of their IgA content by ELISA.The results are shown in Figure 5.

The results show that pLTB proved effective in inducing S-IgA responses at this distant mucosal site. Both B/Harbin and A/Johannesburg antigen responded equally well.

EXAMPLE 7

KINETICS OF IgG RESPONSE

Four groups of eight female BALB/c mice (6-8 weeks) each were treated as follows

Control treated with PBS without antigen. 20 μl i.n. without anaesthesia on days 0, 7 and 14

pLTB 5 μg HA and 2.0 μg recombinant pLTB in 20 μl applied i.n. without anaesthesia on days 0, 7 and 14

HA s.c. 5 μg HA in 100 μl applied s.c. without anaesthesia on day 0

Conv. convalescent mice, i.e. mice infected with 10^8 infective units of PR8 virus, in 20 μl applied i.n. without anaesthesia on day 0

From four mice of each group blood samples were taken from the tail veins on day 6, 13 and 20. Furthermore , on day 28 all mice were sacrificed and bled. In each sample serum IgG was measured by ELISA.

The results are shown in Figure 6. The bars (from left to right) for each of the vaccination regimens represent the IgG titres on days 6, 13, 20 and 28, respectively. These results show that after i.n. vaccination with HA/pLTB the IgG induction is of at least the same magnitude as after s.c. vaccination with HA alone, or as in convalescent mice.

EXAMPLE 8

NASAL AND LUNG MUCOSAL ANTIBODIES

The same mice which were studied in EXAMPLE 7 underwent mucosal lavages of the nasal cavity and the urogenital tract after being sacrificed on day 28 as described above.

The results are summarised in Figure 8. The hatched bars represent the data from nasal washes and the open bars show the data from vaginal washes.

The results indicate that the titre of the first line of defence antibodies (S-IgA) upon i.n. vaccination with HA/pLTB is of at least the same magnitude as the S-IgA titre in

convalescent mice whereas the (classical) s.c. vaccination with HA does not lead to a detectable mucosal IgA titre.

EXAMPLE 9

PROTECTION OF VACCINATED MICE AGAINST CHALLENGE

Four mice of each of the groups of EXAMPLE 7 were infected on day 28 with $5x10^6$ infective units of PR8 virus i.n. in 20 µl without anaesthesia.
Three days post-challenge virus load was determined in nose and lungs.

Virus titration in nose and lung homogenates was carried out on MDCK cells which were cultured on EPISERF (Life Technologies, PAISLY, Scotland) in microtitration plates by two-step dilutions, and by subsequent endpoint determination using haemagglutination with guinea pig erythocytes.

The results are summarised in Figure 7. The hatched bars represent the virus titres in the nose and the open bars are for the lungs. The virus titres in the lungs for convalescent mice and upon vaccination with pLTB were insignificant. Hence, these data show that by using pLTB as a mucosal adjuvant, protection against influenza infection is complete.

CLAIMS

1. Vaccine containing at least one particulate immunogen and an adjuvanting amount of B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli*.

2. Vaccine according to claim 1, wherein the LTB is free from contaminating A subunits or holotoxin.

3. Vaccine according to claim 1-2, wherein the LTB is prepared by recombinant DNA methods.

4. Vaccine according to claim 1-3, wherein viral or bacterial or fungal antigens are used as an immunogen.

5. Vaccine according to claim 1-3, wherein the immunogen provides immunisation against a disease which is transmitted by mucosal infection.

6. Vaccine according to claim 5, wherein influenza antigens are used as an immunogen.

7. Method for the induction of a systemic immunoglobulin response against an immunogen by mucosal administration of said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli*.

8. Method for the induction of a common mucosal immune response against an immunogen by mucosal administration of said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli*.

9. Use of the B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli* in the preparation of a vaccine comprising a particulate immunogen and an adjuvanting amount of said LTB suitable for the induction of a systemic

immunoglobulin response against said immunogen in an individual upon mucosal administration.

10. Use of the B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli* in the preparation of a vaccine comprising a particulate immunogen and an adjuvanting amount of said LTB suitable for the induction of a common mucosal immune response against said immunogen in an individual upon local mucosal administration.

FIGURE 1

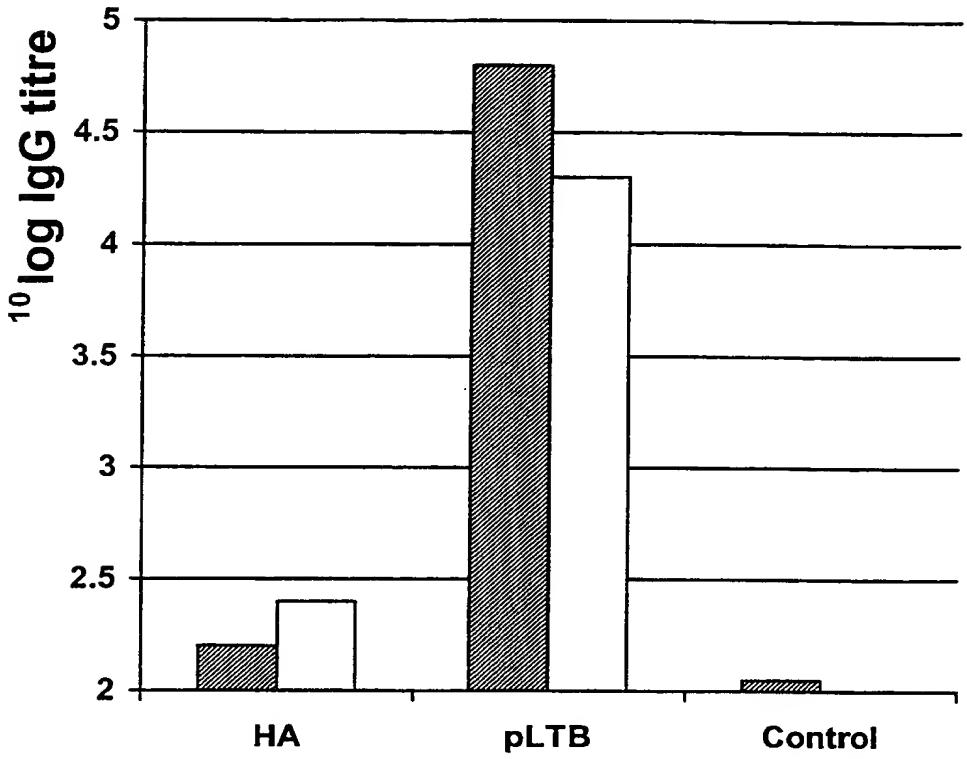


FIGURE 2

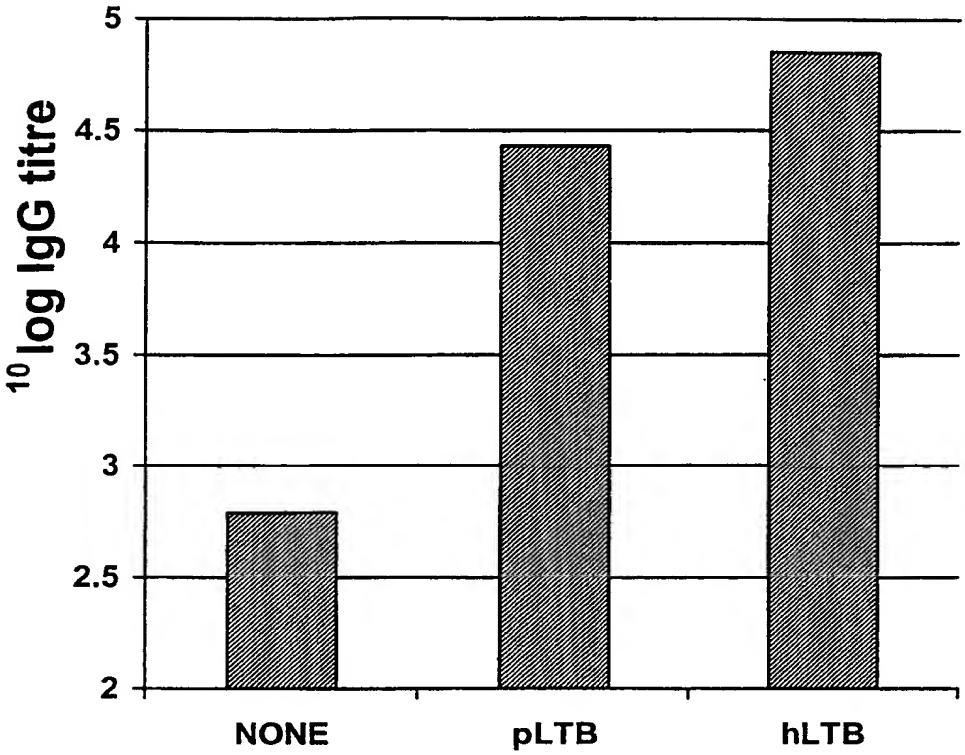


FIGURE 3

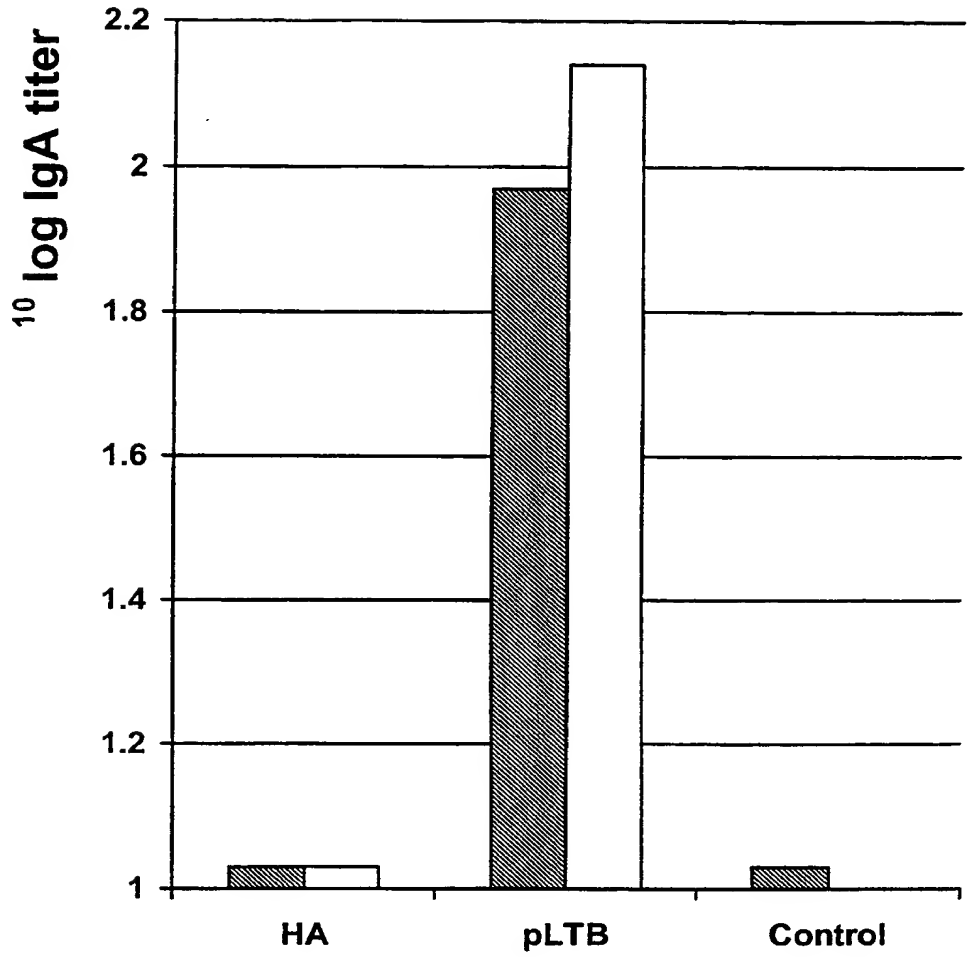


FIGURE 4

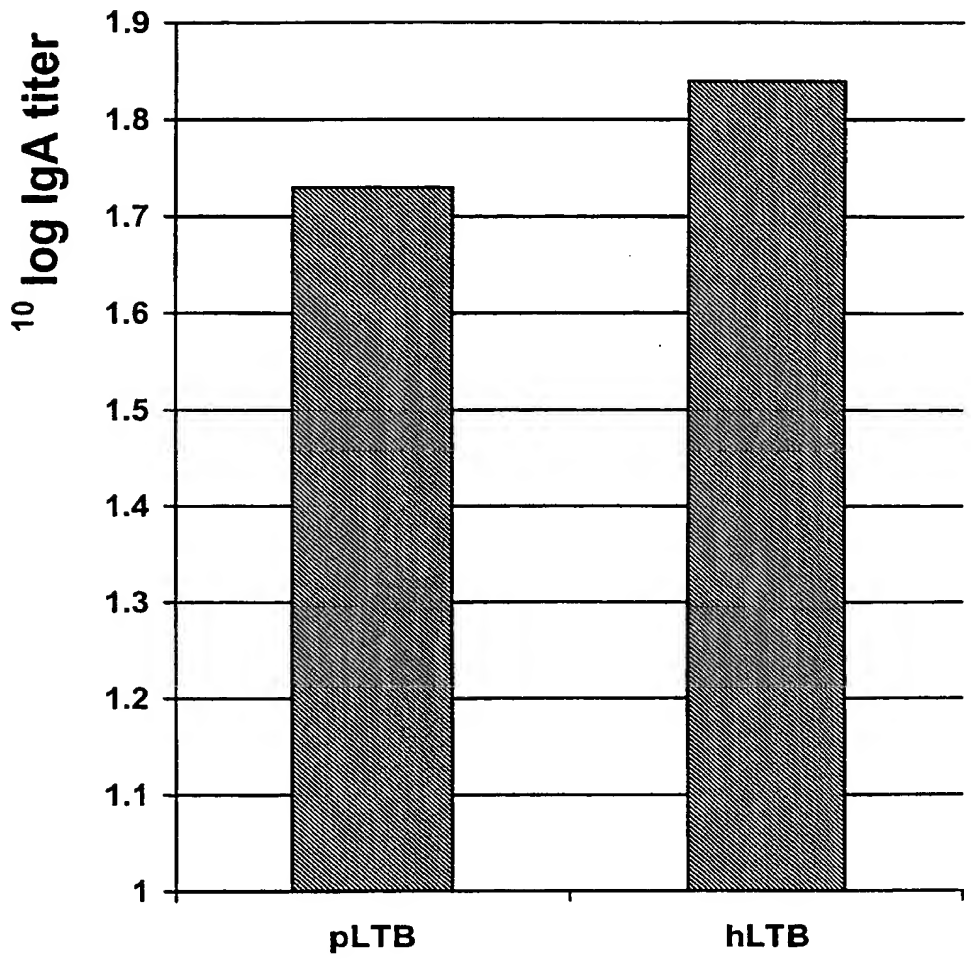


FIGURE 5

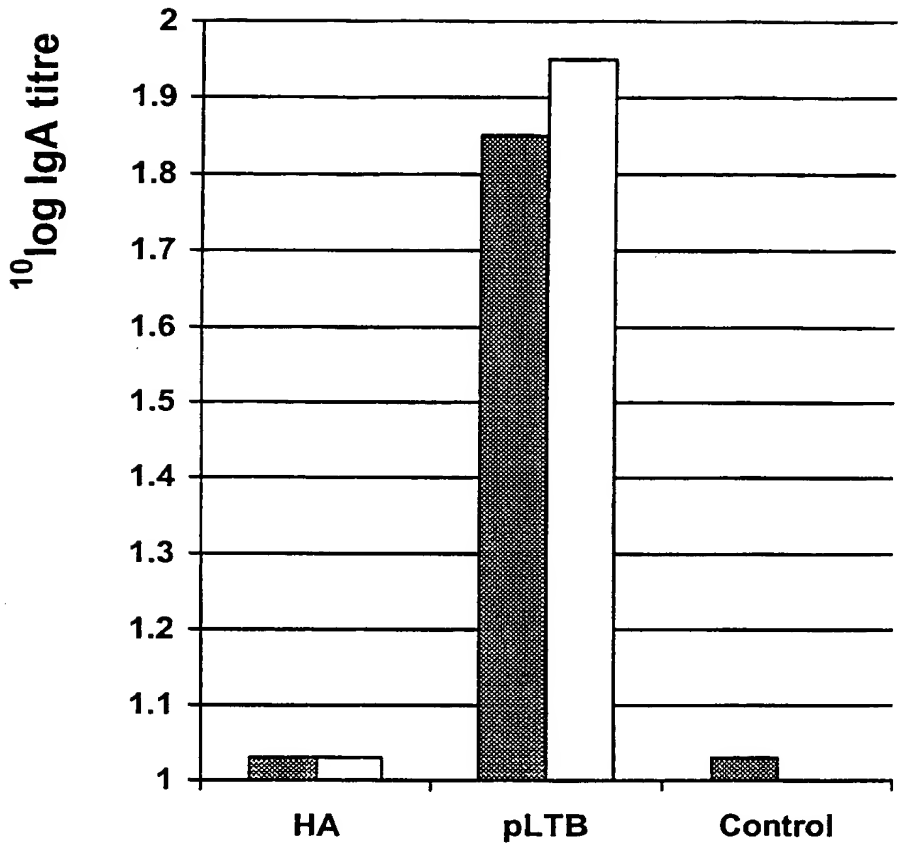


FIGURE 6

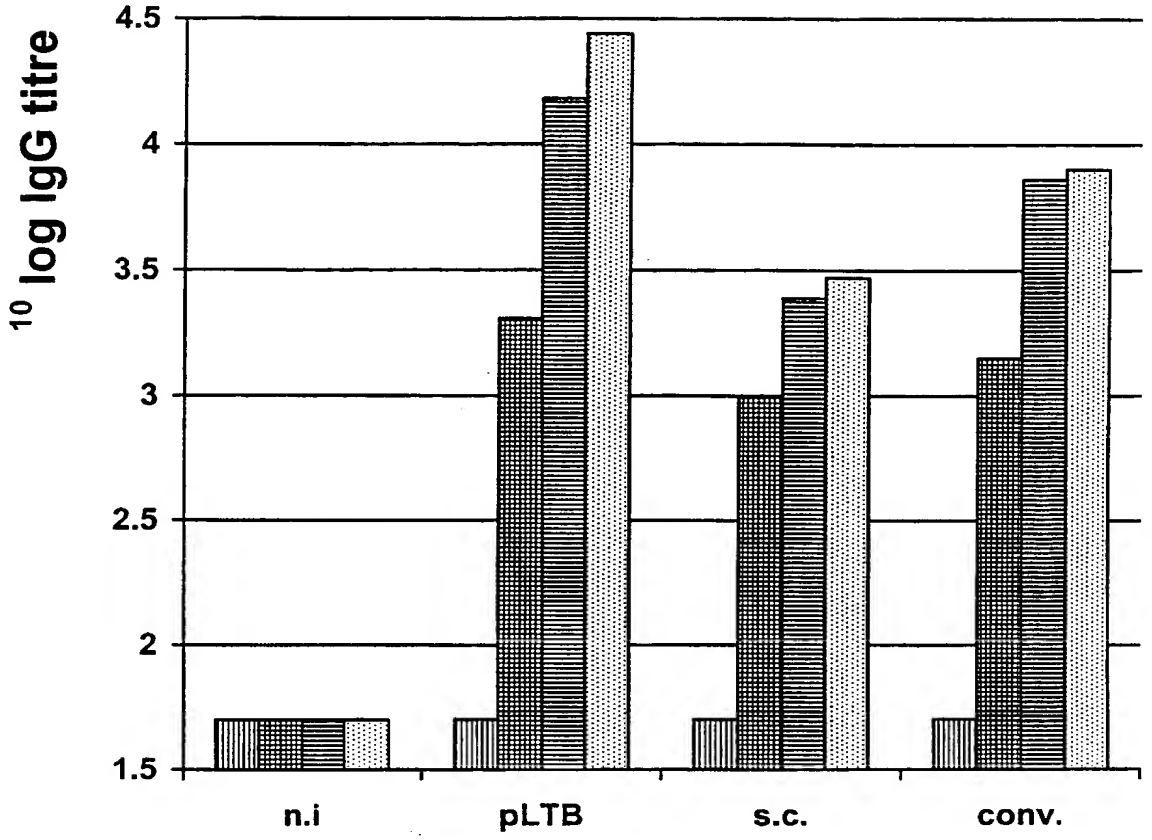


FIGURE 7

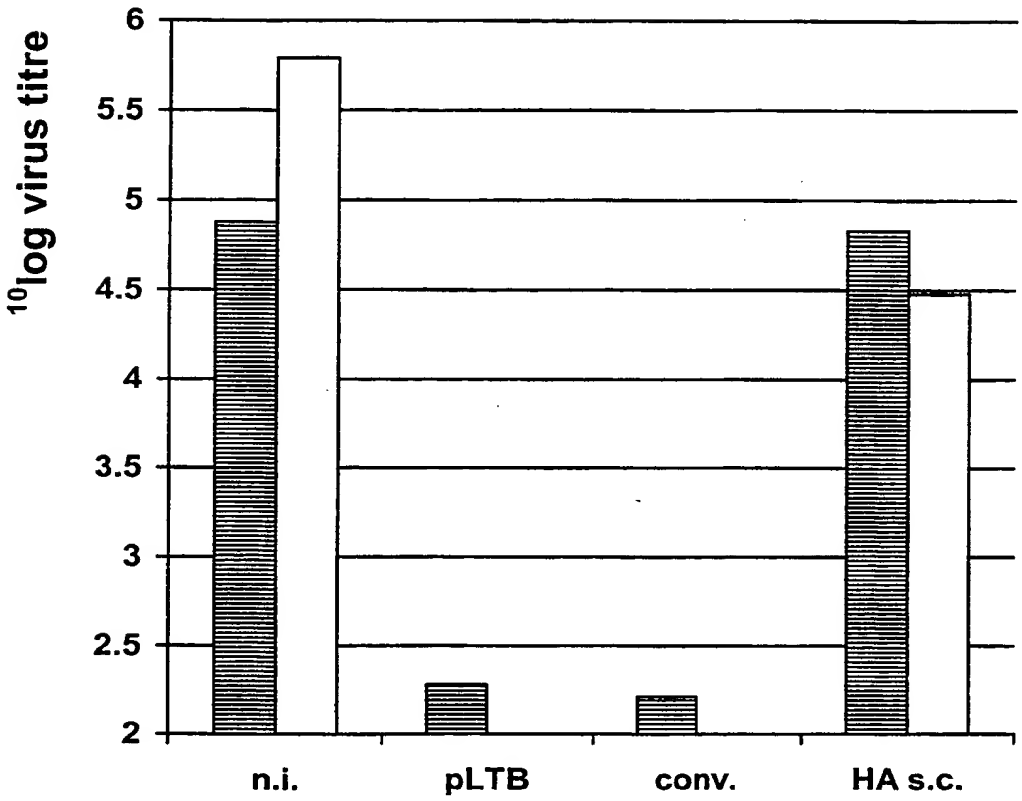
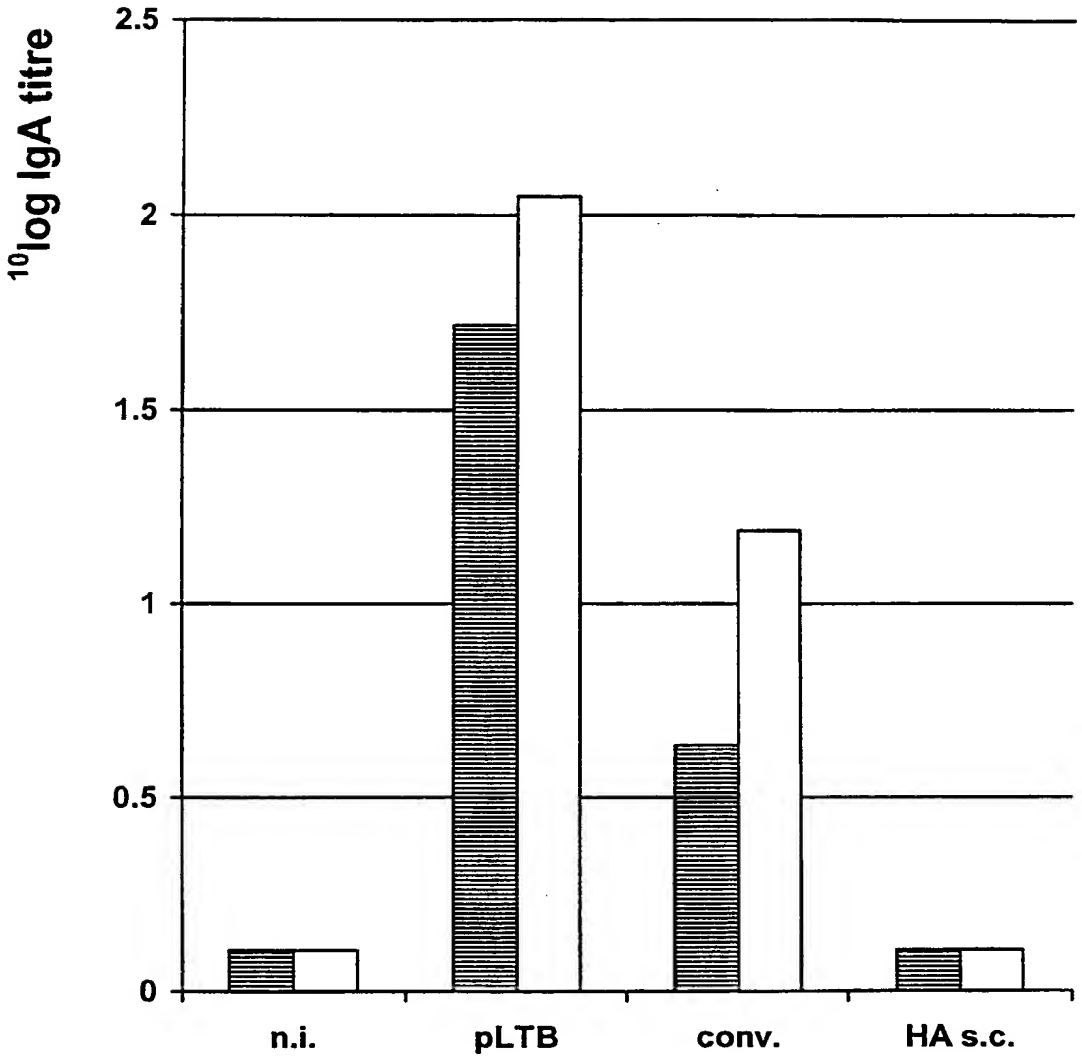


FIGURE 8



CLAIMS

1. Vaccine containing at least one particulate immunogen and an adjuvanting amount of B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli*.

2. Vaccine according to claim 1, wherein the LTB is free from contaminating A subunits or holotoxin.

3. Vaccine according to claim 1-2, wherein the LTB is prepared by recombinant DNA methods.

4. Vaccine according to claim 1-3, wherein viral or bacterial or fungal antigens are used as an immunogen.

5. Vaccine according to claim 1-3, wherein the immunogen provides immunisation against a disease which is transmitted by mucosal infection.

6. Vaccine according to claim 5, wherein influenza antigens are used as an immunogen.

7. Method for the induction of a systemic immunoglobulin response against an immunogen by mucosal administration of said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli*.

8. Method for the induction of a common mucosal immune response against an immunogen by mucosal administration of said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli*.

9. Use of the B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli* in the preparation of a vaccine comprising a particulate immunogen and an adjuvanting amount of said LTB suitable for the induction of a systemic

immunoglobulin response against said immunogen in an individual upon mucosal administration.

10. Use of the B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli* in the preparation of a vaccine comprising a particulate immunogen and an adjuvanting amount of said LTB suitable for the induction of a common mucosal immune response against said immunogen in an individual upon local mucosal administration.

Annexes (amended sheets) to the Preliminary Examination Report

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
DIR 0549		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP98/07553	24/11/1998	25/11/1997
International Patent Classification (IPC) or national classification and IPC A61K39/39		
Applicant DUPHAR INTERNATIONAL RESEARCH B.V. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Auth rity and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Auth rity (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
07/05/1999	19. 10. 99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Mennessi r, T Telephone No. +49 89 2399 8687

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP98/07553

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-12	as originally filed				

Claims, No.:

1-9	as received on	30/09/1999	with letter of	28/09/1999	

Drawings, sheets:

1/8-8/8	as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 6-9.

because:

☒ the said international application, or the said claims Nos. 6-9 (with respect to industrial applicability) r late to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1--9
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-9
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-5
	No:	Claims	

2. Citations and explanations

see separate sheet

1). Comments with respect to item III

a) Claims 6-9 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

b) For the assessment of such claims on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

2). Comments with respect to item V

a) The use of B subunits of the heat-labile enterotoxin from E. Coli when completely free of A subunit or toxic LT holotoxin as an adjuvant in vaccines containing at least one particulate immunogen for mucosal administration appears not to be disclosed in any of the prior art documents cited in the international search report.

b) Furthermore, there is no suggestion in the said documents, whatever taken alone or in combination, that strong systemic and local antibody responses with high level of secretory IgA would be obtained upon mucosal administration of a particulate immunogen adjuvanted with such B subunits.

c) Therefore, it can be considered that the subject-matter of claims 1-9 as a whole is new and involves an inventive step, in accordance with Article 33(2) and (3) PCT.

CLAIMS

1. Vaccine for mucosal administration containing at least one particulate immunogen and an adjuvanting amount of B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli.*, completely free of A subunit or toxic LT holotoxin.

2. Vaccine according to claim 1, wherein the LTB is prepared by recombinant DNA methods.

3. Vaccine according to claim 1-2, wherein viral or bacterial or fungal antigens are used as an immunogen.

4. Vaccine according to claim 1-3, wherein the immunogen provides immunisation against a disease which is transmitted by mucosal infection.

5. Vaccine according to claim 4, wherein influenza antigens are used as an immunogen.

6. Method for the induction of a systemic immunoglobulin response against an immunogen by mucosal administration of said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli*, completely free of A subunit or toxic LT holotoxin.

7. Method for the induction of a common mucosal immune response against an immunogen by mucosal administration of said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli*, completely free of A subunit or toxic LT holotoxin.

8. Use of the B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli* , completely free of A subunit or toxic LT holotoxin in the preparation of a vaccine comprising a particulate immunogen and an adjuvanting amount of said LTB suitable for the induction of a systemic immunoglobulin response against said immunogen in an individual upon mucosal administration.

9. Use of the B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli* , completely free of A subunit or toxic LT holotoxin in the preparation of a vaccine comprising a particulate immunogen and an adjuvanting amount of said LTB suitable for the induction of a common mucosal immune response against said immunogen in an individual upon local mucosal administration.

CLAIMS

1. Vaccine for mucosal administration containing at least one particulate immunogen and an adjuvanting amount of B subunits of heat-labile enterotoxin (LTB) characteristic of *E. coli.*, completely free of A subunit or toxic LT holotoxin.

2. Vaccine according to claim 1, wherein the LTB is prepared by recombinant DNA methods.

3. Vaccine according to claim 1-2, wherein viral or bacterial or fungal antigens are used as an immunogen.

4. Vaccine according to claim 1-3, wherein the immunogen provides immunisation against a disease which is transmitted by mucosal infection.

5. Vaccine according to claim 4, wherein influenza antigens are used as an immunogen.

6. Method for the induction of a systemic immunoglobulin response against an immunogen by mucosal administration of said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli*, completely free of A subunit or toxic LT holotoxin.

7. Method for the induction of a common mucosal immune response against an immunogen by mucosal administration of said immunogen in a particulate form and an adjuvanting amount of B subunits of heat-labile enterotoxin characteristic of *E. coli*, completely free of A subunit or toxic LT holotoxin.

3. Der Gegenstand des vorliegenden Anspruchs 1 unterscheidet sich von D1 dadurch, daß Anspruch 1 ein Verfahren definiert, worin: i) zwei Sensoren benutzt werden, ii) zu erst die Umgebungstemperatur vom gelieferten Signal und einer Zwischenfunktion bestimmt wird, iii) und dann die Körpertemperatur vom Signal des Strahlungssensors und einer Umkehrfunktion der in ii) berechneten Zwischenfunkion bestimmt wird.

   Da keines der vorliegenden Dokumente die in Anspruch 1 definierte Berechnung der Temperatur beschreibt, bei der die Signale von zwei Sensoren, eine Zwischenfunktion und ihre Umkehrfunktion benutzt werden, beruht der Gegenstand des Anspruchs 1 auf einer erfinderischen Tätigkeit (Artikel 33 PCT).

4. Der Vorrichtungsanspruch 6 definiert eine Vorrichtung zur Durchführung des Verfahrens nach Anspruch 1 und erfüllt damit ebenfalls die Erfordernisse des PCT in bezug auf Neuheit und erfinderische Tätigkeit (Artikel 33 PCT) aus den selben Gründen wie in Anspruch 1.

5. Die Ansprüche 2 - 5 sind vom Anspruch 1 abhängig und erfüllen damit ebenfalls die Erfordernisse des PCT in bezug auf Neuheit und erfinderische Tätigkeit.

TENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
DIR 0549		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 98/07553	24/11/1998	25/11/1997
Applicant		
DUPHAR INTERNATIONAL RESEARCH B.V. et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readble form.

[ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

VACCINES WITH A LTB ADJUVANT

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

national application No.
PCT/EP 98/07553

B x I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 7-8 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the vaccine composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

B x II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/07553

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/39 //A61K39:145

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE HAAN, LOLKE ET AL: "Mucosal immunogenicity of the Escherichia coli heat-labile enterotoxin: role of the A subunit" VACCINE (1996), 14(4), 260-266 CODEN: VACCDE;ISSN: 0264-410X, XP004057320 see the whole document ---	1-10
A	HASHIGUCCI, KAZUHIRO ET AL: "Antibody responses in volunteers induced by nasal influenza vaccine combined with Escherichia coli heat-labile enterotoxin B subunit containing a trace amount of the holotoxin" VACCINE (1996), 14(2), 113-19 CODEN: VACCDE;ISSN: 0264-410X, XP004057352 see the whole document --- -/--	1-10

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 April 1999	07.05.99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mennessier, T

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TAMURA, SHIN-ICHI ET AL: "Escherichia coli heat-labile enterotoxin B subunits supplemented with a trace amount of the holotoxin as an adjuvant for nasal influenza vaccine" VACCINE (1994), 12(12), 1083-9 CODEN: VACCDE;ISSN: 0264-410X, XP002100633 cited in the application see the whole document ---	1-10
A	NASHAR T O ET AL: "Current progress in the development of the B subunits of cholera toxin an Escherichia coli heat-labile enterotoxin as carriers for the oral deliver of heterologous antigens and epitopes." VACCINE, (1993) 11 (2) 235-40. REF: 49 JOURNAL CODE: X60. ISSN: 0264-410X., XP000645274 ENGLAND: United Kingdom see the whole document ---	1-10
T	VERWEIJ, WILLEM R. ET AL: "Mucosal immunoadjuvant activity of recombinant Escherichia coli heat-labile enterotoxin and its B subunit: induction of systemic IgG and secretory IgA responses in mice by intranasal immunization with influenza virus surface antigen" VACCINE (DECEMBER 1998), 16(20), 2069-2076 CODEN: VACCDE;ISSN: 0264-410X, XP004138458 see the whole document -----	1-10